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(71) Applicants (for all designated States except US): **VI-RAGEN INCORPORATED** [US/US]; 865 SW 78th Avenue, Suite 100, Plantation, FL 33324 (US). **OXFORD BIOMEDICA PLC** [GB/GB]; Medawar Centre, Oxford Science Park, Oxford OX4 4GA (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SANG, Helen** [GB/GB]; 43 Cluny Drive, Edinburgh EH10 6DU (GB). **MCGREW, Michael** [US/GB]; 6, 2F1 Jameson Place, Edinburgh EH6 8PB (GB). **SHERMAN, Adrian** [GB/GB]; 50 Inverleith Row, Edinburgh EH3 5PX (GB). **JERVIS, Karen, Elizabeth** [GB/GB]; Muirend Cottage, Drumshoreland, Broxburn EH52 5PD (GB). **STIMSON, William, Howard** [GB/GB]; 7 Lawn Park, Fairways, Milngavie, Glasgow G62 6HG (GB). **MITROPHANOUS, Kyriacos** [GR/GB]; Medawar Centre, Oxford Science

Park, Oxford OX4 4GA (GB). **ELLARD, Fiona** [GB/GB]; Medawar Centre, Oxford Science Park, Oxford OX4 4GA (GB). **KINGSMAN, Alan** [GB/GB]; Medawar Centre, Oxford Science Park, Oxford OX4 4GA (GB).

(74) Agent: **MURGITROYD & COMPANY**; Scotland House, 165-169 Scotland Street, Glasgow G5 8PL (GB).

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(54) Title: **PROTEIN PRODUCTION IN TRANSGENIC AVIANS**

(57) Abstract: The present invention relates to a method for the production of transgenic avians using a lentivirus vector system to deliver exogenous genetic material. The invention also relates to the production of proteins by the transgenic avians, preferably in egg whites and a method of testing the likelihood of expression in avian eggs.

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1 **"Protein Production in Transgenic Avians"**

2

3 The present invention relates to the generation of
4 transgenic avians and the production of recombinant
5 proteins. More particularly, the invention relates
6 to the enhanced transduction of avian cells by
7 exogenous genetic material so that the genetic
8 material is incorporated into an avian genome in
9 such a way that the modification becomes integrated
10 into the germline and results in expression of the
11 encoded protein within the avian egg.

12

13 The ability to manufacture large amounts of
14 pharmaceutical grade proteins is becoming
15 increasingly important in the biotechnology and
16 pharmaceutical arenas. Recent successes of such
17 products in the marketplace, especially those of
18 monoclonal antibodies, have put an enormous strain
19 on already stretched global manufacturing
20 facilities. This heightened demand for
21 manufacturing capacity, the consequential high
22 premiums on capacity and the long wait for

1 production space, plus the cost of and issues
2 involved in producing proteins in cell lines, has
3 prompted companies to look beyond traditional modes
4 of production (Andersson & Myhanan, 2001).
5 Traditional methods for manufacture of recombinant
6 proteins include production in bacterial or
7 mammalian cells. One of the alternative
8 manufacturing strategies is the use of transgenic
9 animals and plants for production of proteins.

10

11 It was by genetic engineering that the first
12 genetically modified (transgenic) animal was
13 produced, by transferring the gene for the protein
14 of interest into the target animal. Current
15 transgenic technology can be traced back to a series
16 of pivotal experiments conducted between 1968 and
17 1981 including: the generation of chimeric mice by
18 blastocyst injection of embryonic stem cells
19 (Gardner, 1968), the delivery of foreign DNA to
20 rabbit oocytes by spermatozoa (Brackett et al,
21 1971), the production of transgenic mice made by
22 injecting viral DNA into pre-implantation
23 blastocysts (Jaenisch & Mintz, 1974) and germline
24 transmission of transgenes in mouse by pronuclear
25 injection (Gordon & Ruddle, 1981). For the early
26 part of transgenics' history, the focus was upon
27 improving the genetic makeup of the animal and thus
28 the yield of wool, meat or eggs (Curtis & Barnes,
29 1989; Etches & Gibbins, 1993). However in recent
30 years there has been interest in utilising
31 transgenic systems for medical applications such as
32 organ transplantation, models for human disease or

1 for the production of proteins destined for human
2 use.

3

4 A number of protein based biopharmaceuticals have
5 been produced in the milk of transgenic mice,
6 rabbits, pigs, sheep, goats and cows at reasonable
7 levels, but such systems tend to have long
8 generation times - some of the larger mammals can
9 take years to develop from the founder transgenic to
10 a stage at which they can produce milk. Additional
11 difficulties relate to the biochemical complexity of
12 milk and the evolutionary conservation between
13 humans and mammals, which can result in adverse
14 reactions to the pharmaceutical in the mammals which
15 are producing it (Harvey et al, 2002).

16

17 There is increasing interest in the use of chicken
18 eggs as a potential manufacturing vehicle for
19 pharmaceutically important proteins, especially
20 recombinant human antibodies. Huge amounts of
21 therapeutic antibodies are required by the medical
22 community each year, amounts which can be kilogram
23 or metric tons per year, so a manufacturing
24 methodology which could address this shortage would
25 be a great advantage. Once optimised, a
26 manufacturing method based on chicken eggs has
27 several advantages as compared to mammalian cell
28 culture or use of transgenic mammalian systems.
29 Firstly, chickens have a short generation time (24
30 weeks), which would allow transgenic flocks to be
31 established rapidly. The following table shows a
32 comparison between the different types of transgenic

1 systems. Secondly, the capital outlays for a
2 transgenic animal production facility are far lower
3 than that for cell culture. Extra processing
4 equipment is minimal in comparison to that required
5 for cell culture (BioPharm, 2001). As a consequence
6 of these lower capital outlays, the production cost
7 per unit of therapeutic will be lower than that
8 produced by cell culture. In addition, transgenic
9 systems provide significantly greater flexibility
10 regarding purification batch size and frequency and
11 this flexibility may lead to further reduction of
12 capital and operating costs in purification through
13 batch size optimisation. The third advantage of
14 increased speed to market should become apparent
15 when the technology has been developed to a
16 commercially viable degree. Transgenic mammals are
17 capable of producing several grams of protein
18 product per litre of milk, making large-scale
19 production commercially viable (Weck, 1999).
20 Mammals do not have a significant advantage in terms
21 of the time take to scale up production, since
22 gestation periods for cows and goats are 9 months
23 and 5 months respectively (Dove, 2000) and it can
24 take up to five years to produce a commercially
25 viable herd. However, once the herd is established,
26 the yield of product from milk will be high.

Animal	Gestation	Maturity/ Generation time	Offspring Produced	Time to Production Herd/Flock	Protein (per litre/ egg per day)	Founder animal development cost
Cow	9 months	2 years	1 per year	5+ years	15g	\$5-10M
Goat	5 months	8 months	2-4 per year	3-5 years	8g	\$3M
Sheep	5 months	8 months	2 per year	3-5 years	8g	\$2M
Pigs	4 months	8 months	10	?	4.1g	?
Rabbits	1 month	5 months	8	?	0.05g	?
Chicken	21 days	6 months	21 per month	18 months	0.3g	\$0.25M

A comparison between the various transgenic animal production systems (Dove, 2000).

1 The short generation time for birds also allows for
2 rapid scale-up. The incubation period of a chicken
3 is only 21 days and it reaches maturity within six
4 months of hatch. Indeed, once the founder animals
5 of the flock have been established, a flock can be
6 established within 18 months (Dove, 2000). The
7 process of scaling up the production capability
8 should be simpler and far faster than a herd of
9 sheep, goats or cows.

10

11 A further advantage rests in the fact that eggs are
12 naturally sterile vessels. One of the inherent
13 problems with cell culture methods of production is
14 the risk of microbial contamination, since the
15 nutrient rich media used tends to encourage
16 microbial growth. Transgenic production offers a
17 lower risk alternative, since the production of the
18 protein will occur within the animal itself, whose

1 own body will combat most infections. Chicken eggs
2 provide an even lower risk alternative: the eggs are
3 sealed within the shell and membrane and thus
4 largely separated from the environment. The
5 evolutionary distance between humans and birds means
6 that few diseases are common to both.

7
8 Still a further potential advantage lies in the
9 post-translational modification of chicken proteins.
10 The issue of how well a production process can
11 reproduce the natural sugar profile on the proteins
12 which are produced, is now recognised as a crucial
13 element of the success of a production technology
14 (Parekh *et al*, 1989; Routier *et al*, 1997; Morrow,
15 2001; Raju *et al*, 2000, 2001). The main cell types
16 used in cell culture processes are either hamster or
17 mouse-derived, so do not produce the same sugar
18 pattern on proteins as human cells (Scrip, June 8th
19 2001). Mammalian and particularly plant transgenic
20 systems produce different types of post-
21 translational modifications on expressed proteins.
22 The sugar profile is crucially important to the
23 manner in which the human immune system reacts to
24 the protein. Raju *et al*, (2000) found that
25 glycosylated chicken proteins have a sugar profile
26 that is more similar to that of glycosylated human
27 proteins than non-human mammalian proteins, which
28 should be a significant advantage in developing a
29 therapeutic product.

30
31 It can therefore be seen that the avian egg,
32 particularly from the chicken, offers several major

1 advantages over cell culture as a means of
2 production and the other transgenic production
3 systems based upon mammals or plants.
4 Direct application of the methods used in the
5 production of transgenic mammals to the genetic
6 manipulation of birds has not been possible because
7 of specific features of the reproductive system of
8 the laying hen. Following either natural or
9 artificial insemination, hens will lay fertile eggs
10 for approximately 10 days. They ovulate once per
11 day, and fertilisation occurs almost immediately,
12 while the ovum is at the top of the oviduct. The egg
13 spends the next 20-24 hours in the oviduct, where
14 the albumen (egg white) is laid down around the
15 yolk, plumping fluid is added to the albumen and
16 finally the shell membranes and the shell itself are
17 laid down. During this time, cell division is rapid,
18 such that by the time the egg is laid, the embryo
19 comprises a blastoderm, a disc of approximately
20 60,000 relatively undifferentiated cells, lying on
21 the yolk.
22
23 The complexities of egg formation make the earliest
24 stages of chick-embryo development relatively
25 inaccessible. Methods employed to access earlier
26 stage embryos usually involve sacrificing the donor
27 hen to obtain the embryo or direct injection into
28 the oviduct. Methods for the production of
29 transgenic mammals have focused almost exclusively
30 on the microinjection of a fertilised egg, whereby a
31 pronucleus is microinjected *in vitro* with DNA and
32 the manipulated eggs are transferred to a surrogate

1 mother for development to term, this method is not
2 feasible in hens. Four general methods for the
3 creation of transgenic avians have been developed.
4 A method for the production of transgenic chickens
5 using DNA microinjection into the cytoplasm of the
6 germinal disk was developed. The chick zygotes are
7 removed from the oviduct of laying hens before the
8 first cleavage division, transferred to surrogate
9 shells, manipulated and cultured through to hatch
10 (Perry, 1988; Roslin US 5,011,780 and EP0295964).
11 Love et al, (1994) analysed the embryos that
12 survived for at least 12 days in culture and showed
13 that approximately half of the embryos contained
14 plasmid DNA, with 6% at a level equivalent to one
15 copy per cell. Seven chicks, 5.5% of the total
16 number of ova injected, survived to sexual maturity.
17 One of these, a cockerel identified as a potential
18 mosaic transgenic bird, transmitted the transgene to
19 3.4% of his offspring. These birds have been bred to
20 show stable transmission of the transgene. As in
21 transgenic mice generated by pro-nuclear injection,
22 integration of the plasmid DNA is apparently a
23 random event. However, direct DNA microinjection
24 into eggs results in low efficiencies of transgene
25 integration (Sang & Perry, 1989). It has been
26 estimated that only 1% of microinjected ova give
27 rise to transgenic embryos and of these 10% survive
28 to hatch. The efficiency of this method could be
29 improved by increasing the survival rate of the
30 cultured embryos and the frequency of chromosomal
31 integration of the injected DNA.
32

1 A second method involves the transfection of
2 primordial germ cells *in vitro* and transplantation
3 into a suitably prepared recipient. Successful
4 transfer of primordial germ cells has been achieved,
5 resulting in production of viable gametes from the
6 transferred germ cells. Transgenic offspring, as a
7 result of gene transfer to the primordial germ cells
8 before transfer, have not yet been described.

9

10 The third method involves the use of gene transfer
11 vectors derived from oncogenic retroviruses. The
12 early vectors were replication competent (Salter,
13 1993) but replication defective vectors have been
14 developed (see, eg. US Patent 5,162,215 and WO
15 97/47739). These systems use either the
16 reticuloendotheliosis virus type A (REV-A) or avian
17 leukosis virus (ALV). The efficiency of these
18 vectors, in terms of production of founder
19 transgenic birds, is low and inheritance of the
20 vector from these founders is also inefficient
21 (Harvey et al, 2002). These vectors may also be
22 affected by silencing of expression of the
23 transgenes they carry as reports suggest that
24 protein expression levels are low (Harvey et al,
25 2002).

26

27 The fourth method involves the culture of chick
28 embryo cells *in vitro* followed by production of
29 chimeric birds by introduction of these cultured
30 cells into recipient embryos (Pain et al, 1996). The
31 embryo cells may be genetically modified *in vitro*
32 before chimera production, resulting in chimeric

1 transgenic birds. No reports of germline
2 transmission from genetically modified cells are
3 available.

4
5 Although much work has been carried out on
6 retroviral vectors derived from viruses such as ALV
7 and REV as mentioned previously, the limitations of
8 such vectors have prevented more widespread
9 application. Much of the research and development
10 of viral vectors was based on their use in gene
11 therapy applications and so resulted in the
12 demonstration that vectors based on lentiviruses
13 were able to infect nondividing cells, a clear
14 advantage in clinical gene therapy applications.
15 Lentiviruses are a subgroup of the retroviruses
16 which include a variety of primate viruses eg. human
17 immunodeficiency viruses HIV-1 and 2 and simian
18 immunodeficiency viruses (SIV) and non-primate
19 viruses (eg. maedi-visna virus (MVV), feline
20 immunodeficiency virus (FIV), equine infectious
21 anemia virus (EIAV), caprine arthrithis encephalitis
22 virus (CAEV) and bovine immunodeficiency virus
23 (BIV). These viruses are of particular interest in
24 development of gene therapy treatments, since not
25 only do the lentiviruses possess the general
26 retroviral characteristics of irreversible
27 integration into the host cell DNA, but as mentioned
28 previously, also have the ability to infect non-
29 proliferating cells. The dependence of other types
30 of retroviruses on the cell proliferation status has
31 somewhat limited their use as gene transfer
32 vehicles. The biology of lentiviral infection can

1 be reviewed in Coffin et al, (1997) and Sanjay et
2 al, (1996).

3

4 An important consideration in the design of a viral
5 vector is the ability to be able to stably integrate
6 into the genome of cells. Previous work has shown
7 that oncoretroviral vectors used as gene transfer
8 vehicles have had somewhat limited success due to
9 the gene silencing effects during development.
10 Jahner et al, (1982) showed that use of the vector
11 based on the Moloney murine leukemia virus (MoMLV)
12 for example, is unsuitable for production of
13 transgenic animals due to silencing of the virus
14 during the developmental phase, leading to very low
15 expression of the transgene. It is therefore
16 essential that any viral vector used for production
17 of transgenic birds does not exhibit gene silencing.
18 The work of Pfeifer et al, (2002) and Lois et al,
19 (2002) on mice has shown that a lentiviral vector
20 based on HIV-1 is not silenced during development.

21

22 The bulk of the developmental work on lentiviral
23 vectors has been focused upon HIV-1 systems, largely
24 due to the fact that HIV, by virtue of its
25 pathogenicity in humans, is the most fully
26 characterised of the lentiviruses. Such vectors
27 tend to be engineered as to be replication
28 incompetent, through removal of the regulatory and
29 accessory genes, which render them unable to
30 replicate. The most advanced of these vectors have
31 been minimised to such a degree that almost all of

1 the regulatory genes and all of the accessory genes
2 have been removed.

3

4 The lentiviral group have many similar
5 characteristics, such as a similar genome
6 organisation, a similar replication cycle and the
7 ability to infect mature macrophages (Clements &
8 Payne, 1994). One such lentivirus is Equine
9 Infectious Anemia Virus (EIAV). Compared with the
10 other viruses of the lentiviral group, EIAV has a
11 relatively simple genome: in addition to the
12 retroviral gag, pol and env genes, the genome only
13 consists of three regulatory/accessory genes (tat,
14 rev and S2). The development of a safe and
15 efficient lentiviral vector system will be dependent
16 on the design of the vector itself. It is important
17 to minimise the viral components of the vector,
18 whilst still retaining its transducing vector
19 function. A vector system derived from EIAV has been
20 shown to transduce dividing and non-dividing cells
21 with similar efficiencies to HIV-based vectors
22 (Mitrophanous et al, 1999). Oncoretroviral and
23 lentiviral vectors systems may be modified to
24 broaden the range of transducible cell types and
25 species. This is achieved by substituting the
26 envelope glycoprotein of the virus with other virus
27 envelope proteins. These include the use of the
28 amphotropic MLV envelope glycoprotein (Page et al,
29 1990), the baculovirus GP64 envelope glycoprotein
30 (Kumar et al, 2003), the adenovirus AD5 fiber
31 protein (Von Seggern et al, 2000) rabies G-envelope
32 glycoprotein (Mazarakis et al, 2001) or the

1 vesicular stomatitis virus G-protein (VSV-G) (Yee et
2 al, 1994). The use of VSV-G pseudotyping also
3 results in greater stability of the virus particles
4 and enables production of virus at higher titres.

5

6 It is an aim of the present invention to provide an
7 efficient method for transferring a transgene
8 construct to avian embryonic cells so as to create a
9 transgenic bird which expresses the gene in its
10 tissues, especially, but not exclusively, in the
11 cells lining the oviduct so that the translated
12 protein becomes incorporated into the produced eggs.

13

14 It is also an aim of the present invention to
15 provide a vehicle and a method for transferring a
16 gene to avian embryonic cells so as to create a
17 transgenic bird which has stably incorporated the
18 transgene into a proportion or all of its germ
19 cells, resulting in transmission of the transgene to
20 a proportion of the offspring of the transgenic
21 bird. This germ line transmission will result in a
22 proportion of the offspring of the founder bird
23 exhibiting the altered genotype.

24

25 It is a further aim of the present invention to
26 provide an efficient method for genetic modification
27 of avians, enabling production of germ line
28 transgenic birds at high frequency and reliable
29 expression of transgenes.

30

31 According to the present invention there is provided
32 a method for the production of transgenic avians,

1 the method comprising the step of using a lentivirus
2 vector system to deliver exogenous genetic material
3 to avian embryonic cells or cells of the testes.

4

5 The lentivirus vector system includes a lentivirus
6 transgene construct in a form which is capable of
7 being delivered to and integrated with the genome of
8 avian embryonic cells or cells of the testes.

9

10 Preferably the lentivirus vector system is delivered
11 to and integrated at an early stage of development
12 such as early cleavage when there have only been a
13 few cell divisions.

14

15 In one embodiment the lentivirus transgene construct
16 is injected into the subgerminal cavity of the
17 contents of an opened egg which is then allowed to
18 develop.

19

20 The Perry Culture system of surrogate shells may be
21 used.

22

23 Alternatively methods used by Bosselmann et al. or
24 Speksnijder and Ivarie of windowing of the egg can
25 be used. In these methods an embryo in a newly laid
26 egg may be accessed by cutting a window in the egg
27 shell and injecting the lentivirus vector system
28 into the embryonic subgerminal cavity. The egg may
29 then be sealed and incubated.

30

31 In another embodiment the construct is injected
32 directly into the sub-blastodermal cavity of an egg.

1

2 Typically the genetic material encodes a protein.

3

4 The genetic material may encode for any of a large
5 number of proteins having a variety of uses
6 including therapeutic and diagnostic applications
7 for human and/or veterinary purposes and may include
8 sequences encoding antibodies, antibody fragments,
9 antibody derivatives, single chain antibody
10 fragments, fusion proteins, peptides, cytokines,
11 chemokines, hormones, growth factors or any
12 recombinant protein.

13

14 The invention thus provides a transgenic avian.

15

16 Preferably the transgenic avian produced by the
17 method of the invention has the genetic material
18 incorporated into at least a proportion of germ
19 cells such that the genetic material will be
20 transmitted to at least a proportion of the
21 offspring of the transgenic avian.

22

23 The invention also provides the use of a lentivirus
24 vector system in the production of a transgenic
25 avian.

26

27 It has been surprisingly observed that the use of
28 lentiviral transgene constructs described by the
29 present invention transduce germ cells of avian
30 embryos with unexpectedly high efficiency.
31 Resulting avians subsequently transmit the
32 integrated vector to a high proportion of offspring

1 and the transgene carried by the vector may be
2 expressed at relatively high levels.

3

4 The invention thus provides further transgenic
5 avians.

6

7 According to the present invention there is also
8 provided a method for production of an heterologous
9 protein in avians, the method comprising the step of
10 delivering genetic material encoding the protein
11 within a lentivirus vector construct to avian
12 embryonic cells so as to create a transgenic avian
13 which expresses the genetic material in its tissues.

14

15 Preferably the transgenic avian expresses the gene
16 in the oviduct so that the translated protein
17 becomes incorporated into eggs.

18

19 The protein can then be isolated from eggs by known
20 methods.

21

22 The invention provides the use of a lentivirus
23 construct for the production of transgenic avians.

24

25 The invention also provides the use of a lentivirus
26 vector construct for the production of proteins in
27 transgenic avians.

28

29 Preferably the lentivirus vector construct is used
30 for the expression of heterologous proteins in
31 specific tissues, preferably egg white or yolk.

32

1 The lentivirus as used in this application may be
2 any lentiviral vector but is preferably chosen from
3 the group consisting of EIAV, HIV, SIV, BIV and FIV.

4
5 A particularly preferred vector is EIAV.

6
7 Any commercially available lentivirus vector may be
8 suitable to be used as a basis for a construct to
9 deliver exogeneous genetic material.

10
11 Preferably the construct includes suitable enhancer
12 promoter elements for subsequent production of
13 protein.

14
15 A specific promoter may be used with a lentiviral
16 vector construct to result in tissue specific
17 expression of the DNA coding sequence. This may
18 include promoters such as CMV, pCAGGS or any
19 promoter based upon a protein usually expressed in
20 an avian egg, such as ovalbumin, lysozyme,
21 ovotransferrin, ovomucoid, ovostatin, riboflavin-
22 binding protein or avidin.

23
24 Preferably the vector construct particles are
25 packaged using a commercially available packaging
26 system to produce vector with an envelope, typically
27 a VSV-G envelope.

28
29 Typically the vector may be based on EIAV available
30 from ATCC under accession number VR-778 or other
31 commercially available vectors.

32

1 Commercial lentivirus-based vectors for use in the
2 methods of the invention are capable of infecting a
3 wide range of species without producing any live
4 virus and do not cause cellular or tissue toxicity.

5

6 The methods of the present invention can be used to
7 generate any transgenic avian, including but not
8 limited to chickens, turkeys, ducks, quail, geese,
9 ostriches, pheasants, peafowl, guinea fowl, pigeons,
10 swans, bantams and penguins.

11

12 These lentivirus-based vector systems also have a
13 large transgene capacity which are capable of
14 carrying larger protein encoding constructs such as
15 antibody encoding constructs.

16

17 A preferred lentiviral vector system is the
18 LentiVector® system of Oxford BioMedica.

19

20 The invention further provides a method to determine
21 the likelihood of expression of a protein in vivo,
22 the method comprising the step of measuring
23 expression of the protein in avian oviduct cells in
24 vitro.

25

26 The invention therefore provides the use of avian
27 cells in vitro to determine the likelihood of
28 expression in vivo.

29

30 The invention is exemplified with reference to the
31 following non-limiting experiments and with
32 reference to the accompanying drawings wherein:

1
2 Figure 1 illustrates a schematic representation of
3 the EIAV vectors used in this study.

4 Figure 2 illustrates Southern transfer analysis of
5 genomic DNA from individual birds to identify
6 proviral insertions.

7
8 Figure 3 illustrates reporter gene expression in
9 pONY8.0cZ and pONY8.0G G₁ transgenic birds.

10
11 Figure 4 illustrates reporter gene expression in
12 pONY8.4GCZ G₁ transgenic birds.

13
14 Figure 5 illustrates reporter gene expression in G₂
15 transgenic birds.

16
17 Figure 6 illustrates Western analysis of pONY8.4GCZ
18 G₁ birds.

19
20 Figure 7 illustrates reporter gene expression in
21 pONY8.0cZ G₂ birds.

22
23 Figure 8 illustrates lacZ expression in the oviduct
24 of a transgenic bird.

25
26 Experiment 1

27
28 Freshly laid, fertile hen's eggs were obtained which
29 contain developing chick embryos at developmental
30 stages X-XIII (Eyal-Giladi & Kochav, 1976). An egg
31 was opened, the contents transferred to a dish and

1 2-3 microlitres of a suspension of lentiviral vector
2 virus particles was injected into the subgerminal
3 cavity, below the developing embryo but above the
4 yellow yolk. The vector used was derived from Equine
5 Infectious Anaemia Virus (EIAV) and carried a
6 reporter gene, β -galactosidase (lacZ), under the
7 control of the CMV (cytomegalovirus)
8 enhancer/promoter. The packaging system used to
9 generate the vector viral particles resulted in
10 production of the vector with a VSV-G envelope. The
11 estimated concentration of viral transducing
12 particles was between 5×10^7 and 1×10^9 per ml. The
13 embryos were allowed to develop by culturing them
14 using the second and third phases of the Perry
15 culture system (Perry, 1988). 12 embryos were
16 removed and analysed for expression of lacZ after 2
17 days of incubation and 12 embryos after 3 days of
18 incubation. The embryos and surrounding membranes
19 were dissected free of yolk, fixed and stained to
20 detect expression of the lacZ reporter gene. All
21 embryos showed expression of lacZ in some cells of
22 the embryo and surrounding membranes. The expression
23 was highest in the developing extraembryonic
24 membrane close to the embryo and was limited to a
25 small number of cells in the embryos analysed. These
26 results indicated that all the embryos had been
27 successfully transduced by the injected lentiviral
28 vector.

29

30 Experiment 2

31

1 In a further experiment 40 laid eggs were injected
2 each with 2-3 microlitres of a suspension of the
3 EIAV vector at a titre of 5×10^8 per ml., into the
4 sub-blastodermal cavity. 13 chicks hatched (33%) and
5 were screened to identify transgenic offspring
6 carrying the lentiviral vector sequence. Samples of
7 the remaining extraembryonic membrane were recovered
8 from individual chicks after hatch, genomic DNA
9 extracted and the DNA analysed by PCR using primers
10 specific to the lentiviral vector sequence. The
11 screen identified 11 chicks as transgenic (85%). The
12 vector sequence was detected in the extraembryonic
13 membrane at a copy number of between 0.4% and 31%,
14 indicating that the chicks were mosaic for
15 integration of the vector. This result was predicted
16 as the embryos were injected with the vector at a
17 stage at which they consisted of at least 60,000
18 cells. It is unlikely that all the cells in the
19 embryo would be transduced by the viral vector,
20 resulting in chicks that were chimeric for
21 integration of the vector. The 11 chicks were raised
22 to sexual maturity and 7 found to be males. Semen
23 samples were obtained from the cockerels when they
24 reached 16-20 weeks of age. DNA from these samples
25 was screened by PCR and the seven cockerels found to
26 have lentiviral vector sequence in the semen at
27 levels estimated as between 0.1% and 80%. The
28 majority of the samples contained vector sequence at
29 a level above 10%. This suggested that at least 10%
30 of the offspring of these cockerels will be
31 transgenic. Semen was collected from one cockerel,
32 code no. LEN5-20, that had been estimated to have a

1 copy number of the viral vector in DNA from a blood
2 sample as 6%. The copy number estimated from the
3 semen sample was 80%. The semen was used to
4 inseminate stock hens, and the fertile eggs
5 collected and incubated. 9 embryos were recovered
6 after 3 days of incubation, screened by PCR to
7 identify transgenic embryos and stained for
8 expression of the lacZ reporter gene. 3 of the 9
9 embryos were transgenic and all 3 expressed lacZ but
10 at a very low level in a small number of cells. 12
11 embryos were recovered after 10 days of incubation
12 and screened as above. 6 embryos were identified as
13 transgenic and lacZ expression detected in 4. The
14 expression was high in several tissues in one embryo
15 and lower in the other 3. These results indicate
16 that 43% of the offspring of cockerel LEN5-20 were
17 transgenic. The expression of the reporter construct
18 carried by the lentiviral vector varied between
19 individual transgenic chicks. It is likely that the
20 individual chicks had copies of the vector genome
21 integrated at different chromosomal sites, which may
22 affect the expression of the transgene. It is also
23 possible that some chicks carried more than one copy
24 of the transgene.

25
26 The results outlined here demonstrate that a
27 specific EIAV-derived lentiviral vector, pseudotyped
28 with the VSV envelope protein, can transduce the
29 germ cells of chick embryos with very high
30 efficiency. The resulting birds then transmit the
31 integrated vector to a high proportion of their
32 offspring. The transgene carried by the vector may

1 be expressed to give a functional protein at
2 relatively high levels. The transgene carried by the
3 vector may be designed to express foreign proteins
4 at high levels in specific tissues.

5

6 The lentiviral vector may be introduced into the
7 chick at different developmental stages, using
8 modifications of the method described in the example
9 above.

10

11 The viral suspension may be injected above the
12 blastoderm embryo in a new laid egg .

13 The viral suspension may be injected into the newly
14 fertilised egg or the early cleavage stages, up to
15 stageX (Eyal-Giladi & Kochav, 1976), by utilizing
16 the culture method of Perry (1988) or recovering
17 eggs from the oviduct and then returning them to a
18 recipient hen by ovum transfer.

19

20 The viral suspension may be injected above or below
21 the blastoderm embryo in a freshly laid egg which
22 has been accessed by cutting a window in the shell.
23 The window may be resealed and the egg incubated to
24 hatch (Bosselman et al, 1989).

25

26 The viral suspension may be injected into the testes
27 of cockerels and semen screened to detect
28 transduction of the spermatogonia and consequent
29 development of transgenic sperm.

30

31 Experiment 3

32

1 Materials and Methods

2

3 EIAV vectors and preparation of virus stocks

4 The vectors pONY8.0cZ and pONY8.0G have been
5 described previously (Pfeifer et al, 2002). The
6 vector pONY8.4GCZ has a number of modifications
7 including alteration of all ATG sequences in the
8 gag-derived region to ATTG, to allow expression of
9 eGFP downstream of the 5'LTR. The 3' U3 region has
10 been modified to include the Moloney leukaemia virus
11 U3 region. Vector stocks were generated by FuGENE6
12 (Roche, Lewes, U.K.) transfection of HEK 293T cells
13 plated on 10cm dishes with 2µg of vector plasmid,
14 2µg of gag/pol plasmid (pONY3.1) and 1µg of VSV-G
15 plasmid (pRV67) (Lois et al, 2002). 36-48 hours
16 after transfection supernatants were filtered
17 (0.22µm) and stored at -70°C. Concentrated vector
18 preparations were made by initial low speed
19 centrifugation at 6,000xg for 16 hours at 4°C
20 followed by ultracentrifugation at 50,500xg for 90
21 minutes at 4°C. The virus was resuspended in
22 formulation buffer (Lois et al, 2002) for 2-4 hours,
23 aliquoted and stored at -80°C.

24

25 Production and analysis of transgenic birds

26 Approximately 1-2µl of viral suspension was
27 microinjected into the sub-germinal cavity beneath
28 the blastodermal embryo of new-laid eggs. Embryos
29 were incubated to hatch using phases II and III of
30 the surrogate shell ex vivo culture system (Challita
31 & Kohn, 1994). DNA was extracted from the CAM of
32 embryos that died in culture at or after more than

1 twelve days of development using Puregene genomic
2 DNA purification kit (Flowgen, Asby de la Zouche,
3 U.K.). Genomic DNA samples were obtained from CAM of
4 chicks at hatch, blood samples from older birds and
5 semen from mature cockerels. PCR analysis was
6 carried out on 50ng DNA samples for the presence of
7 proviral sequence. To estimate copy number control
8 PCR reactions were carried out in parallel on 50ng
9 aliquots of chicken genomic DNA with vector plasmid
10 DNA added in quantities equivalent to that of a
11 single copy gene (1x), a 10-fold dilution (0.1x) and
12 a 100-fold dilution (0.01x) as described previously
13 (Perry, 1988). Primers used:
14 5'CGAGATCCTACAGTTGGCGCCCGAACAG3' and
15 5'ACCAGTAGTTAATTTCTGAGACCCTTGTA-3'. The number of
16 proviral insertions in individual G₁ birds was
17 analysed by Southern transfer. Genomic DNA extracted
18 from whole blood was digested with *Xba*I or *Bam*HI.
19 Digested DNA was resolved on a 0.6%(w/v) agarose gel
20 then transferred to nylon membrane (Hybond-N,
21 Amersham Pharmacia Biotech, Amersham U.K.).
22 Membranes were hybridised with ³²P-labelled probes
23 for the reporter gene *lacZ* or eGFP at 65°C.
24 Hybridisation was detected by autoradiography. All
25 experiments, animal breeding and care procedures
26 were carried out under license from the U.K. Home
27 Office.
28
29 Expression analysis
30 Adult tissues were isolated and fixed for 30 min in
31 4% paraformaldehyde, 0.25% gluteraldehyde, in
32 phosphate buffered saline (PBS). Tissues were cryo-

1 embedded and sectioned at 14 μ m. β -galactosidase
2 activity was detected by incubating at 37°C in 5mM
3 potassium ferricyanide, 5mM potassium ferrocyanide,
4 2mM $MgCl_2$, 0.5mg/ml X-gal for 90 min (sections) or 4
5 hours (embryos). GFP images of hatchlings were
6 captured using Fujifilm digital camera (Nikon 60mm
7 lens) shot through a GFsP-S lens system (BLS, Ltd,
8 Czech Republic). Selected tissues were snap-frozen
9 and total protein was extracted by homogenization in
10 PBS containing protease inhibitors (complete mini,
11 Roche, Lewes, U.K.). Protein concentration was
12 determined by Bradford assay. Either 50 μ g (Fig. 4)
13 or 100 μ g (Fig. 3) of protein extract was resolved
14 on 12% polyacrylamide gels (Invitrogen, Paisley,
15 U.K.) and transferred to PDVF membranes. Membranes
16 were incubated with mouse anti- β -galactosidase
17 antibody (Promega, Southampton, U.K.) at 1:5000
18 dilution and donkey anti-mouse IgG-HRP antibody
19 (Santa Cruz Biotech) at 1:2000 dilution and
20 visualized with the ECL western blotting detection
21 system (Amersham Biosciences, Amersham, U.K.). ELISA
22 was performed using β -gal Elisa kit (Roche, Lewes,
23 U.K.).

24

25 Results

26 Detailed Figure legends

27

28 Figure 1. Schematic representation of the EIAV
29 vectors used in this study.

30 The light grey box represents the EIAV packaging
31 signal, and the diagonal lined box in pONY8.4GCZ the
32 MLV U3 region. Restriction sites (XbaI [X], BstEII

1 [B] utilised for Southern blot analysis are
2 indicated. The reporter gene *lacZ* was used as a
3 probe (Fig. 2).

4
5 Figure 2. Southern transfer analysis of genomic DNA
6 from individual birds to identify proviral
7 insertions. Genomic DNA samples were digested with
8 *Xba*I (a, c, d) or *Bst*EII (b) and hybridised with a
9 probe for *lacZ*. (a, b) Analysis of 14 G1 offspring
10 of G0 bird no. 1-4 (Table 1) revealed multiple
11 proviral insertions in the G1 birds. (c) Analysis of
12 G1 bird no. 2-2/19 (lane 1) and 14 of his G2
13 offspring (lanes 2-15) and (d) G1 bird 2-2/6 (lane
14 1) and 9 of his G2 offspring (lanes 2-10),
15 demonstrated stability of the proviral insertions
16 after germ line transmission.

17
18 Figure 3. Reporter gene expression in pONY8.0cZ and
19 pONY8.0G G₁ transgenic birds.

20 a Western blot analysis of liver, heart, skeletal
21 muscle, brain, oviduct, skin, spleen, intestine,
22 kidney, pancreas and bone marrow protein extracts
23 from 5 adult G₁ birds each containing single,
24 independent insertions of pONY8.0cZ. 100µg of
25 protein was loaded per lane and β-galactosidase
26 protein detected as described in Experimental
27 Protocols. b Sections of skin, pancreas, and
28 intestine from G₁ 2-2/19 stained for β-
29 galactosidase activity and comparable sections of a
30 non-transgenic control bird (arrowheads indicate
31 epidermis of skin, villi of intestine). Bar = 0.5mm.
32 c Sections of breast muscle, pancreas, and skin from

1 a single copy transgenic or a wildtype bird were
2 visualized for GFP fluorescence (arrowhead indicates
3 epidermis of skin). Bar = 0.5mm.

4

5 Figure 4. Reporter gene expression in pONY8.4GCZ G₁
6 transgenic birds.

7 a Sections of tissues from a single copy G₁ bird was
8 stained for β -galactosidase activity (arrow
9 indicates smooth muscle of intestine). Bar = 0.5mm.
10 Panel A: higher magnification of oviduct section.
11 Arrows identify cells lining tubular glands cut in
12 cross-section. Bar = 0.05mm. b Levels of β -
13 galactosidase protein were determined for pONY8.0cZ
14 and pONY8.4GCZ lines. Data points were generated
15 from three independent experiments.

16

17 Figure 5. Reporter gene expression in G₂ transgenic
18 birds.

19 a Western analysis of protein extracted from
20 intestine, skin, liver and pancreas of G₁ cockerels
21 2-2/19 and 2-2/6 and two G₂ offspring of each bird. b
22 Top panel: five G₁ offspring of bird ID 4-1. The 4
23 birds on the left are transgenic for pONY8.0G and
24 express eGFP. The bird on the right is not
25 transgenic. Bottom panel: five G₂ offspring of bird
26 ID 4-1/66. The bird in the center is not transgenic.

27

28 Figure 6. Western analysis of pONY8.4GCZ G₁ birds.
29 Western blot analysis of liver, heart, skeletal
30 muscle, brain, oviduct, skin, spleen, intestine,
31 kidney, pancreas and bone marrow protein extracts
32 from 4 adult G₁ birds each containing single,

1 independent insertions of pONY8.4GCZ. 100µg of
2 protein was loaded per lane and β-galactosidase
3 protein detected as described in Experimental
4 Protocols.

5
6 Figure 7. Reporter gene expression in pONY8.0cZ G2
7 transgenic birds.

8 Sections of skin, pancreas and intestine (arrowhead
9 indicates epidermis, arrow indicates feather
10 follicle) from a G2 offspring of 2-2/19 stained for
11 β-galactosidase activity and comparable sections of
12 a non-transgenic control bird. Bar = 0.5mm

13

14 Production of G₀ transgenic birds

15 Three different self-inactivating EIAV vectors
16 (Fig.1) were used, pseudotyped with vesicular
17 stomatitis virus glycoprotein (VSV-G). These vectors
18 have previously been used to transduce a number of
19 tissues in several animal model systems, both in
20 vitro and in vivo (Pfeifer et al, 2002; Rholl et al,
21 2002; Corcoran et al, 2002; Azzouz et al, 2002). The
22 pONY8.4 vector was modified from pONY8.0 by
23 substitution of Moloney murine leukaemia virus
24 (MoMLV) sequence in the 5' LTR and deletion of the
25 majority of the viral env gene. The vector
26 preparations were concentrated to give titres of
27 approximately 10⁷ to 10¹⁰ transducing units per
28 millilitre (T.U./ml). One to two microlitres of
29 concentrated vector was injected into the
30 subgerminal cavity below the developing embryonic
31 disc of new-laid eggs, which were then cultured to
32 hatch. Genomic DNA was extracted from

1 chorioallantoic membrane (CAM) of hatched G₀ chicks
2 and analysed by PCR to detect the EIAV packaging
3 site sequence. The approximate copy number of the
4 vector with respect to the amount of genomic DNA
5 present was estimated, with a range from the
6 equivalent of one copy per genome to 0.01 copies per
7 genome (see Experimental Protocol). All chicks were
8 raised to sexual maturity and genomic DNA from semen
9 samples from males was similarly screened by PCR.
10
11 Four experiments were carried out. The virus
12 pONY8.0cZ was injected at a titre of 5×10^7 T.U./ml
13 in experiment 3.1 and 5×10^8 T.U./ml in experiment
14 3.2. In experiment 3.3 the virus pONY8.4GCZ was
15 injected at a concentration of 7.2×10^8 T.U./ml and
16 in experiment 3.4 pONY8.0G was used at 9.9×10^9
17 T.U./ml. A total of 73 eggs were injected in the
18 four experiments from which 20 (27%) chicks hatched.
19 The results of the PCR screen of hatched male and
20 female chicks from each experiment are shown in
21 Table 1. Fourteen of the twenty G₀ birds contained
22 vector sequences at levels estimated to be between
23 0.5 to 0.01 copies per genome equivalent. The vector
24 pONY8.0cZ transduced the chick embryos more
25 efficiently than the vector pONY8.4GCZ when injected
26 at a similar concentration, possibly due to the
27 presence of the viral cPPT sequence that is involved
28 in nuclear import of the viral DNA genome (Lois et
29 al, 2002). The results also show that transgenic
30 birds can be produced using titres as low as 5×10^7
31 T.U./ml, but that transduction frequency increases
32 if higher titres are used.

1
2 Germ line transmission from G₀ males
3 Semen samples were collected from the 12 G₀ males
4 when they reached sexual maturity, between 16 and 20
5 weeks of age. The results of PCR screens of genomic
6 DNA extracted from these samples are given in Table
7 1. These showed that vector sequences were present
8 in the germ line of all the cockerels, even those
9 that had been scored as not transgenic when screened
10 at hatch. This was confirmed by breeding from 10 of
11 the 12 cockerels by crossing to stock hens and
12 screening their G₁ offspring to identify transgenic
13 birds. All 10 cockerels produced transgenic
14 offspring, with frequencies ranging from 4% to 45%.
15 The frequencies of germ line transmission were very
16 close to those predicted from the PCR analysis of
17 semen DNA but, in every case, higher than predicted
18 from analysis of DNA from CAM samples taken at
19 hatch. Blood samples were taken from several
20 cockerels and PCR analysis closely matched the
21 results from the CAM DNA analysis (data not shown).
22 The results suggest a germ line transduction
23 frequency approximately 10-fold higher than that of
24 somatic tissues.
25
26 Analysis of G₁ transgenic birds and transmission to
27 G₂
28 The founder transgenic birds were transduced at a
29 stage of development when embryos consist of an
30 estimated 60,000 cells, approximately 50 of which
31 are thought to give rise to primordial germ cells
32 (Bienemann et al, 2003; Ginsburg & Eyal-Giladi,

1 1987). We predicted that the G_1 birds to result from
2 separate transduction events of individual
3 primordial germ cells and that different birds would
4 have independent provirus insertions, representing
5 transduction of single germ cell precursors. It was
6 also possible that individual cells would have more
7 than one proviral insertion. Four G_0 cockerels,
8 transduced with pONY8.0cZ (experiments 3.1 and 3.2),
9 were selected for further analysis of their
10 transgenic offspring (Table 2). Genomic DNA from
11 individual G_1 birds was analysed by Southern blot.
12 Samples were digested separately with *Xba*I and *Bst*
13 *EII*, restriction enzymes that cut within the
14 integrated EIAV provirus but outside the probe
15 region (Fig. 1), and hybridised with probes to
16 identify restriction fragments that would represent
17 the junctions between the proviral insertions and
18 the genomic DNA at integration sites. This enabled
19 estimation of the number of proviral insertions in
20 each G_1 bird and of the number of different
21 insertions present in the offspring of each G_0
22 analysed. An example of this analysis is shown in
23 Fig. 2a,b and the results summarised in Table 2. The
24 majority of G_1 birds carried single proviral
25 insertions but several contained multiple copies,
26 with a maximum of 4 detected in one bird. Some
27 offspring of each G_0 bird carried the same proviral
28 insertion, indicating that they were derived from
29 the same germ cell precursor.
30
31 Three male G_1 offspring of bird 2-2 (2-2/6,16 and
32 19) were crossed to stock hens to analyse

1 transmission frequency to the G_2 generation.
2 Cockerels 2-2/6 and 2-2/19 had single proviral
3 insertions and the ratios of transgenic to non-
4 transgenic offspring, 14/30 (47%) and 21/50 (42%),
5 did not differ significantly from the expected
6 Mendelian ratio. Cockerel 2-2/16 had two proviral
7 insertions and 79% (27/34) of the G_2 offspring were
8 transgenic, reflecting the independent transmission
9 of two insertions. Southern transfer analysis was
10 used to compare the proviral insertion present in
11 birds 2-2/6 and 2-2/19 with 9 and 14 of their G_2
12 offspring, respectively (Fig, 2c,d). Identical
13 restriction fragments were observed in parents and
14 offspring, indicating that the proviruses were
15 stable once integrated into the genome.

16

17 Transgene expression in G_1 and G_2 transgenic birds
18 The vectors pONY8.0cZ and pONY8.4GCZ carried the
19 reporter gene *lacZ* under control of the human
20 cytomegalovirus (CMV) immediate early
21 enhancer/promoter (CMVp) and pONY8.0G carried the
22 reporter eGFP, also controlled by CMVp. Expression
23 of *lacZ* was analysed by staining of tissue sections
24 to detect β -galactosidase activity and by western
25 analysis of protein extracts from selected tissues
26 isolated from adult birds, to identify β -
27 galactosidase protein. Expression of eGFP was
28 analysed using UV illumination.

29

30 Protein extracts were made from a range of tissues
31 from seven pONY8.0cZ G_1 birds, each containing a
32 different single provirus insertion. A protein of

1 the expected 110kDa was detected in some tissues in
2 each transgenic bird. Expression was consistently
3 high in pancreas and lower levels of protein were
4 present in other tissues, including liver, intestine
5 and skeletal muscle. The analysis of five of these
6 birds is shown in Figure 3a. β -galactosidase was
7 detected in most tissues on longer exposures of the
8 western blot (data not shown). The pattern of
9 expression was consistent between the individual
10 birds but the overall amounts of protein varied.
11 Sections of tissues from an adult pONY8.0cZ G_1 bird
12 were stained (Fig. 3b). Intense staining was
13 observed throughout the exocrine pancreas and in
14 other tissues, such as the epithelium of the skin
15 and villi of the small intestine. Expression
16 analysis of GFP in sections of tissue from a
17 pONY8.0G bird detected expression in the pancreas,
18 skin and breast muscle (Fig. 3c) and weak expression
19 in the intestine (data not shown). These results
20 show that transgenic birds produced with the same
21 EIAV vector but carrying different reporter genes
22 showed similar patterns of expression.

23

24 Western analysis of tissues from six G_1 birds
25 carrying different single proviral insertions of
26 pONY8.4GCZ detected lacZ expression in four birds,
27 in a pattern similar to that seen in the pONY8.0cZ
28 transgenic birds (Fig.6). However, staining of
29 tissue sections revealed a more extensive pattern of
30 expression than was observed in birds transgenic for
31 pONY8.0cZ. β -galactosidase activity was detected
32 additionally in the smooth muscle of the intestine,

1 blood vessels underlying the epidermis and in
2 tubular gland cells of the oviduct (Fig. 4a). An
3 ELISA assay was used to quantify the differences in
4 levels of expression of β -galactosidase between
5 transgenic birds carrying the pONY8.0 and pONY8.4
6 vectors (Fig. 4b). β -galactosidase levels were
7 higher in pONY8.4GCZ birds in all tissues assayed
8 than in pONY8.0cZ birds. Levels in pancreatic
9 extracts were approximately 6-fold higher and
10 expression in bird no. 3-5/337 was 30pg per
11 microgram of tissue, or 3% of total protein.

12
13 To establish if transgene expression was maintained
14 after germ line transmission, expression in G_2 birds
15 carrying the vectors pONY8.0cZ and pONY8.0G was
16 examined. Western analysis was carried out on tissue
17 extracts from two G_1 cockerels, 2-2/6 and 2-2/19,
18 that each had a single proviral insertion, and two
19 G_2 offspring from each cockerel (Fig. 5a). β -
20 galactosidase protein levels are very similar in the
21 parent and two offspring and the patterns of
22 expression, predominantly in the pancreas, are also
23 very similar. Staining of tissue sections from a G_2
24 bird demonstrated expression patterns comparable to
25 that observed in the parent (Fig. 7). GFP
26 fluorescence was readily detected in live G_1 chicks
27 carrying pONY8.0G and the G_2 offspring of one of
28 these birds showed a similar level of expression
29 (Fig. 5b).

30
31 Figure 8 shows a range of sections from the oviduct
32 of a transgenic hen carrying the vector pONY8.4GCZ

1 carrying the reporter gene lac Z. Blue stain is
2 apparent in the sections illustrating expression of
3 lacZ.

4
5 Discussion

6 We have demonstrated that the lentiviral vector
7 system that we have tested is a very efficient
8 method for production of germ line transgenic birds.
9 In the experiments described here twelve cockerels
10 were produced after injection of concentrated
11 suspension of viral vector particles immediately
12 below the blastoderm stage embryo in new laid eggs.
13 We bred from ten founder cockerels and all produced
14 transgenic offspring, with frequencies from 4 to
15 45%. Even the lowest frequency of germ line
16 transmission obtained is practical in terms of
17 breeding to identify several G₁ transgenic birds from
18 one founder cockerel, in order to establish
19 independent lines carrying different proviral
20 insertions. This method of sub-blastodermal
21 injection is very similar to the methods used
22 previously (Salter & Crittenden, 1989; Bosselman et
23 al, 1989; Harvey et al, 2002) to introduce
24 retroviruses into the chicken. The high success rate
25 may be due to a number of factors, including the
26 ability of lentiviral vectors to transduce non-
27 dividing cells, the use of the VSV-G pseudotype,
28 that has previously been used to introduce a
29 retroviral vector into quail (Karagenc et al, 1996),
30 and the high titres used compared to previous
31 transgenic studies. The chick embryo in a laid egg
32 is a disc consisting of a single layer of cells,

1 lying on the surface of the yolk, with cells
2 beginning to move through the embryo to form the
3 hypoblast layer below the embryonic disc (Mizuarai
4 et al, 2001). Primordial germ cells also migrate
5 from the embryonic disc, through the subgerminal
6 cavity and on to the hypoblast below. It is possible
7 that during the developmental stages immediately
8 after the virus injection, the primordial germ cells
9 migrate through the suspension of viral particles,
10 thus accounting for the higher frequency of germ
11 cell transduction compared to that of cells of the
12 CAM or blood.

13

14 We have shown that the majority of G_1 transgenic
15 birds contain a single proviral insertion but that
16 some birds contain multiple insertions. These
17 results indicate that it will be easy to use this
18 vector system to generate transgenic birds with
19 single vector-transgene insertions and to breed
20 several lines from the same G_0 bird, with the
21 provirus inserted at different chromosomal loci.
22 Levels of expression of a transgene, introduced by a
23 particular vector but integrated at different sites
24 within the chicken genome, are likely to vary. The
25 analysis of transmission from G_1 to G_2 indicates that
26 it will be simple to establish lines carrying stable
27 transgene insertions, using the lentiviral vectors
28 described.

29

30 Expression of the reporter gene *lacZ* was detected in
31 founder (G_0), G_1 and G_2 birds. The expression of *lacZ*
32 was directed by human CMVp (nucleotides -726 to +

78), an enhancer/promoter generally described as functioning ubiquitously in many cell types. This is usually the case if it is used in cell culture transfection experiments but expression in transgenic mice from the CMVp varies between tissues. In particular, it has been reported that CMVp transgene shows predominant expression in exocrine pancreas in transgenic mice (Eyal-Giladi & Kochav, 1976). We have shown that the pattern of expression of both *lacZ* and GFP in embryos and birds is predominantly in the pancreas, although it is expressed at varying levels in most tissues. Expression from the third generation EIAV vector pONY8.4 was significantly higher than from the pONY8.0 vector, possibly due to increase in mRNA stability in the former resulting from removal of instability elements in the *env* region. Transgene expression was not detected in a small number of pONY8.4GCZ transgenic birds, possibly due to the inclusion of MoMLV sequence in the vector that may induce silencing (Zhan et al, 2000). The expression pattern seen in G₁ birds is maintained after germ line transmission to G₂. These results indicate that transgene-specific expression, from transgenes introduced using lentiviral vectors, is maintained after germ line transmission, as has been described in the mouse and rat (Naldini et al, 1996). The size of transgenes that can be incorporated in lentiviral vectors is limited and therefore some tissue-specific regulatory sequences may be too big for use in these vectors. The limit has yet to be defined but is likely to be up to 8kb, as EIAV vectors of

1 9kb have been successfully produced (Lois et al,
2 2002).

3

4 Expression of lacZ in the oviduct (Fig. 8)
5 demonstrates that the cells which synthesize egg
6 white proteins can express foreign proteins in
7 transgenic birds carrying an integrated lentiviral
8 vector system encoding a protein.

9

10 The study described here is an evaluation of the
11 possible application of lentiviral vectors for the
12 production of transgenic birds. We have shown that
13 we can obtain a very high frequency of germline
14 transgenic birds, stable transmission from one
15 generation to the next, and a pattern of transgene
16 expression that is maintained after germline
17 transmission. These results indicate that the use of
18 lentiviral vectors will overcome many of the
19 problems encountered so far in development of a
20 robust method for production of transgenic birds.
21 The application of this method for transgenic
22 production will allow many transgene constructs to
23 be tested to determine those that express in
24 appropriate tissues and at required levels. Recently
25 an ALV vector has been used to generate a transgenic
26 line in which expression and accumulation in egg
27 white of low amounts of biologically active protein
28 was demonstrated (Rapp et al, 2003). Although the
29 amounts of protein produced, micrograms of protein
30 per egg, is not at a level that will facilitate
31 commercial production, the analysis of the protein
32 purified from egg white supports the aim that

1 transgenic hens may be used as bioreactors. The use
2 of lentiviral vectors may overcome the problems
3 associated with transgene incorporation and
4 expression using oncoretroviral vectors. The
5 development of an efficient method for production of
6 transgenic birds is particularly timely as the
7 chicken genome sequence is due to be completed this
8 year and the value of the chick as a model for
9 analysis of vertebrate gene function is increasing
10 (Mozdziak et al, 2003).

11

12 Experiment 4

13

14 Experiments are being carried out with the
15 Invitrogen ViraPower™ system. The chickenised R24
16 minibody coding sequence is inserted into the
17 pLenti6/V5 plasmid immediately downstream of the
18 constitutive CMV promoter. ViraPower™ 293FT cells
19 are then cotransfected with the pLenti6/V5/R24
20 expression construct and the optimised ViraPower™
21 packaging mix. Finally packaged virus-containing
22 tissue culture supernatant is harvested. One
23 intended use for the Invitrogen ViraPower™ system is
24 as a high efficiency transfection reagent. The
25 presence of the blasticidin resistance gene on the
26 pLenti6/V5 plasmid confers the ability to
27 preferentially select transduced populations. This
28 means relatively low titre viral harvests are
29 adequate. However, for the experimental work
30 described below, more concentrated viral harvests
31 are required. Two methods of viral concentration
32 are being evaluated. First, the use of spin

1 concentration via Centrikon Plus20 spin columns.
2 Second, the use of a standard ultracentrifugation
3 protocol.

4

5 The structure of the RNA genome of the concentrated
6 packaged viral vectors is being analysed by both
7 Northern blotting and Reverse Transcriptase-
8 Polymerase Chain Reaction (RT-PCR). Reverse
9 transcription is carried out with several reverse
10 primers, oligo dT, random hexamers and a primer
11 specific to the 3'LTR, to ensure that a
12 representative sample of viral genomes are converted
13 to cDNA. The integrity of the cR24 coding sequence
14 in the cDNA samples is verified using individual PCR
15 reactions optimised to amplify specific sequences.

16

17 The packaged pLenti6/V5/R24 viral vector is also
18 being used for transduction of 293T cells *in vitro*.
19 Multiple pLenti6/V5/R24 viral dilutions are prepared
20 in standard tissue culture medium with the addition
21 of polybrene. The virus/medium/polybrene mixes are
22 then added to cells. After three hours the tissue
23 culture medium is replenished until after a further
24 72hrs the medium is harvested. The level of
25 secreted cR24 minibody is then quantified via ELISA.
26 Transduced cells are also selected with blasticidin
27 for a period of 7-10 days before medium is
28 harvested. Here also the level of secreted cR24
29 minibody is quantified via ELISA.

30

31 Furthermore, the packaged pLenti6/V5/R24 viral
32 vector is also being used for the transduction of

1 chick embryos *in vivo* via injection into the
2 subgerminal cavity, below the developing embryo but
3 above the yellow yolk.

4

5

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Table 1. PCR analysis of hatched chicks and germline transmission from founder cockerels

Experiment: Construct (Viral titre)	Bird No.	Genome equivalents		Germline transmission Transgenics/total
		CAM	Semen	
1. pONY8.0cZ 5 x 10 ⁷ T.U./ml	1-1	0	0.05	1/14 (7%)
	1-2	0.01	♀	-
	1-3	0	♀	-
	1-4	0.01	0.5	16/55 (29%)
	1-5	0.01	0.1	nd
2. pONY8.0cZ 5 x 10 ⁸ T.U./ml	2-1	0.1	♀	-
	2-2	0.1	1.0	4/20 (20%)
	2-3	0	0.01	nd
	2-4	0.1	0.5	19/67 (28%)
	2-5	0	♀	-
	2-6	0.05	♀	-
	2-7	0.05	♀	-
	2-8	0.05	0.5	15/60 (25%)
3. pONY8.4GCZ 7.2 x 10 ⁸ T.U./ml	3-1	0	0.05	1/25 (4%)
	3-2	0	0.05	3/64 (5%)
	3-3	0.01	♀	-
	3-4	0.01	0.05	4/100 (4%)
	3-5	0.01	0.1	9/82 (11%)
	3-6	0.01	♀	-
4. pONY8.0G 9.9 x 10 ⁹ T.U./ml	4-1	0.05	1.0	20/44 (45%)

Table 2. Estimation of number of provirus insertions in the genome of G₁ birds

Bird no.	Total G ₁ analysed	Number of birds with N insertions				Total no. independent insertions
		1	2	3	4	
1-4	14	11	3	0	0	10
2-2	4	3	1	0	0	4
2-4	14	11	2	1	0	14
2-8	14	10	1	2	1	19

1 Claims

2

3 1 A method for the production of transgenic avians,
4 the method comprising the step of using a lentivirus
5 vector system to deliver exogenous genetic material
6 to avian embryonic cells or cells of the testes.

7

8 2 A method as claimed in claim 1 wherein the
9 lentivirus vector system includes a lentivirus
10 transgene construct in a form which is capable of
11 being delivered to and integrated with the genome of
12 avian embryonic cells or cells of the testes.

13

14 3 A method as claimed in claim 2 wherein the
15 lentivirus construct is injected into the
16 subgerminal cavity of the contents of an opened egg
17 which is then allowed to develop.

18

19 4 A method as claimed in claim 2 wherein the
20 construct is injected directly into the sub-
21 blastodermal cavity of an egg.

22

23 5 A method as claimed in any of the preceding claims
24 wherein the vector construct transduces germ cells
25 at high efficiency.

26

27 6 A method as claimed in any of the preceding claims
28 wherein the genetic material encodes a protein.

29

30 7 A transgenic avian produced by a method as claimed
31 in any of the preceding claims.

32

1 8 A transgenic avian and subsequent transgenic
2 offspring produced as the offspring of a transgenic
3 avian as claimed in claim 7.

4

5 9 A method for the production of an heterologous
6 protein in avians, the method comprising the step of
7 delivering genetic material encoding the protein
8 within a lentivirus vector construct to avian
9 embryonic cells so as to create a transgenic avian
10 which expresses the genetic material in its tissues.

11

12 10 A method as claimed in claim 9 wherein the
13 transgenic avian expresses the gene in the oviduct
14 so that the translated protein becomes incorporated
15 into eggs.

16

17 11 A method as claimed in claim 10 further
18 comprising the step of isolating the protein from
19 the eggs.

20

21 12 Use of a lentivirus construct for the production
22 of transgenic avians.

23

24 13 Use of a lentivirus vector construct for the
25 production of proteins in transgenic avians.

26

27 14 Use as claimed in claim 13 of lentivirus vector
28 construct for the expression of heterologous
29 proteins in specific tissues, preferably egg white
30 or yolk.

31

1 15 Use as claimed in any of claims 12 to 14 wherein
2 the lentivirus is chosen from the group consisting
3 of EIAV, HIV, SIV, BIV and FIV.

4

5 16 Use as claimed in any of claims 12 to 15 wherein
6 the construct includes suitable enhancer promoter
7 elements for subsequent production of protein.

8

9 17 Use as claimed in any of claims 12 to 16 wherein
10 the vector construct particles are packaged to
11 produce vector with an envelope.

12

13 18 A method of determining the likelihood of
14 expression of a protein in a transgenic avian, the
15 method comprising the step of detecting expression
16 of the protein in oviduct cells in vitro.

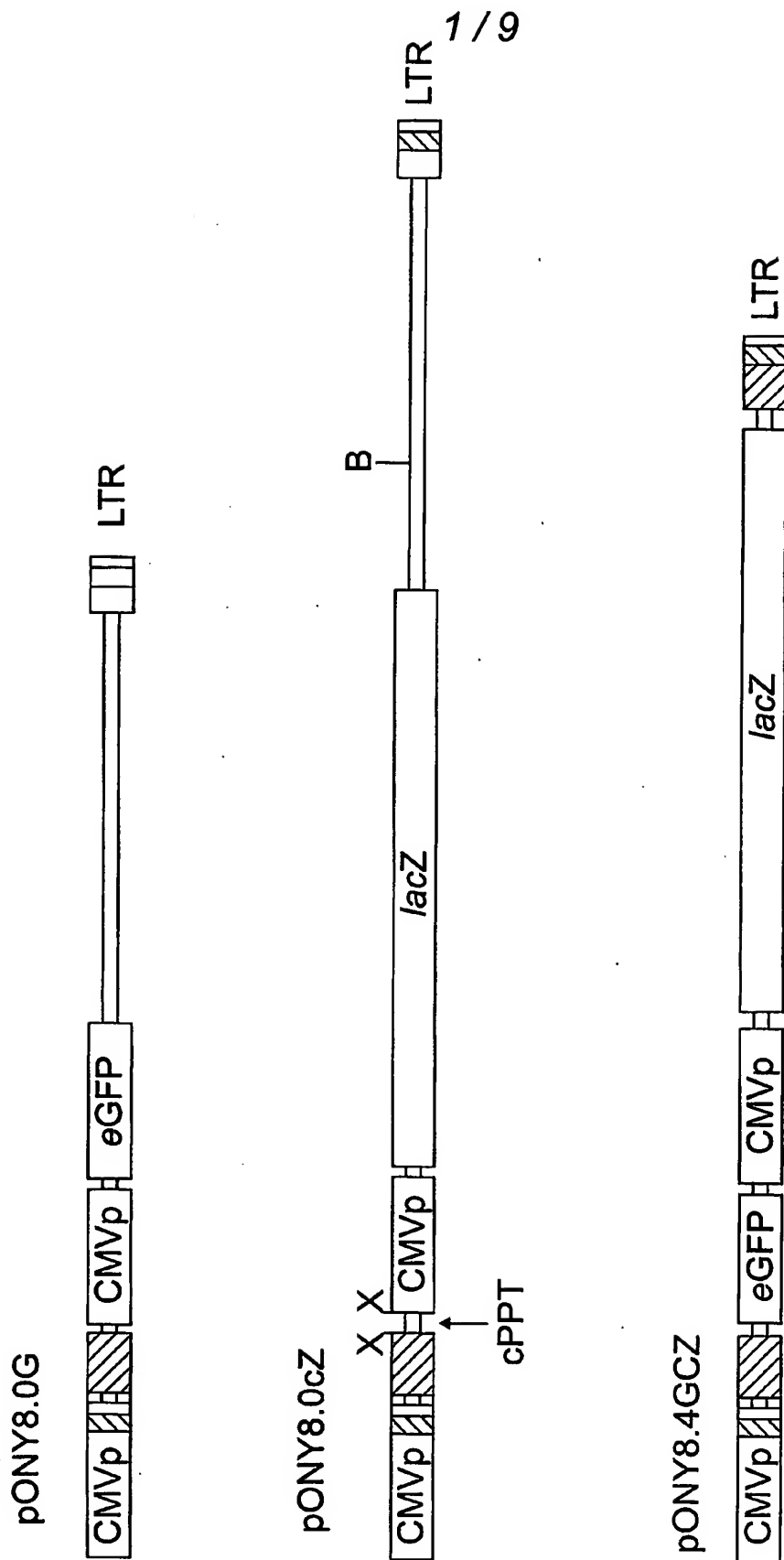
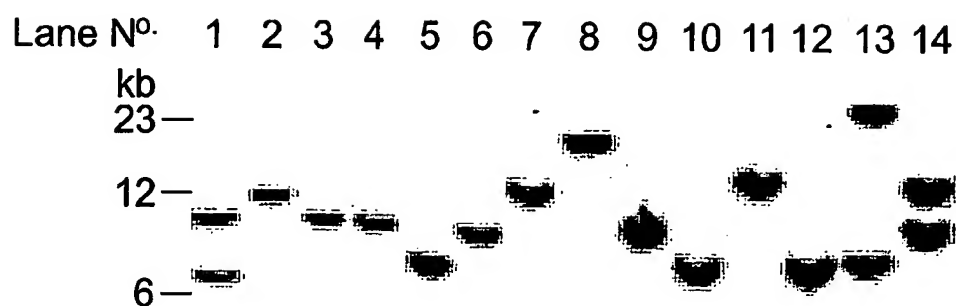
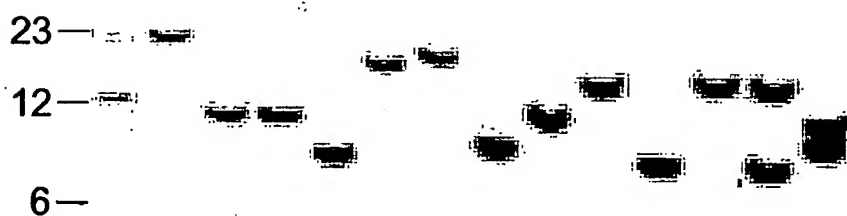
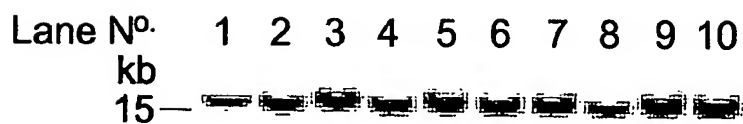
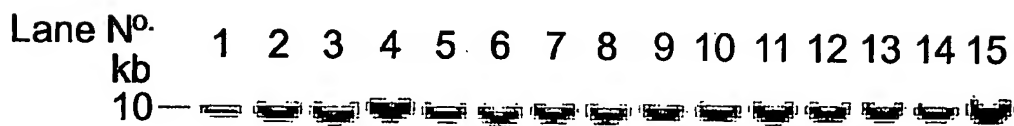


Fig. 1

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*Fig. 2a**Fig. 2b**Fig. 2c**Fig. 2d*

3 / 9

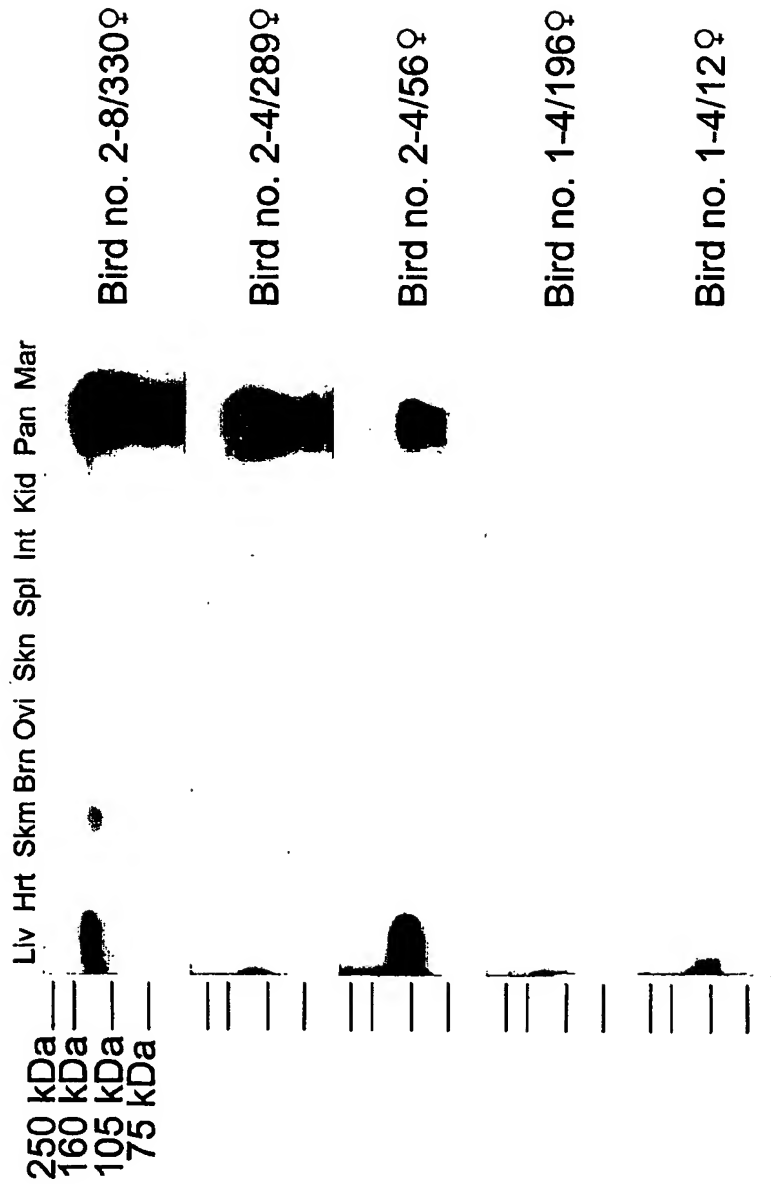
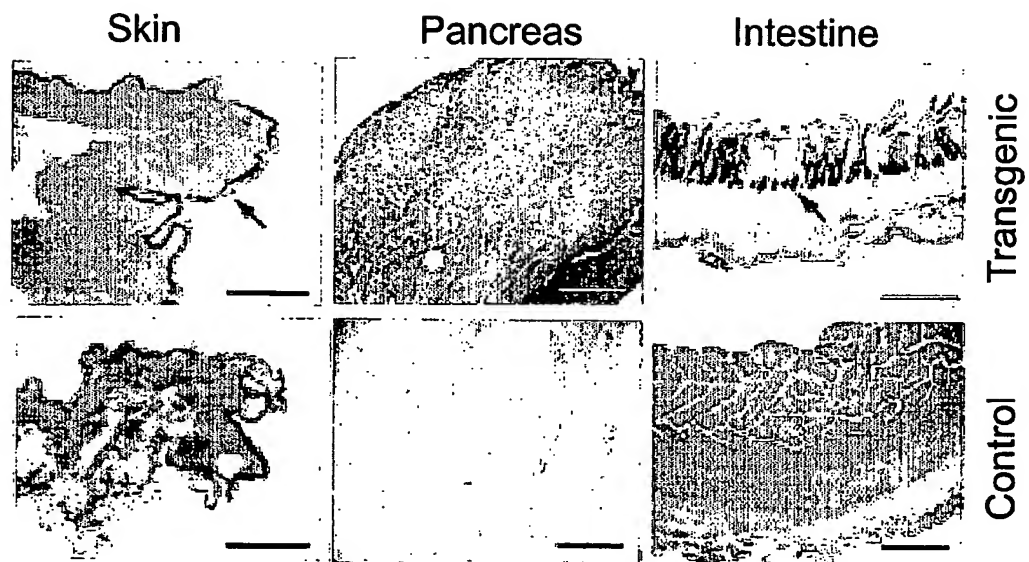
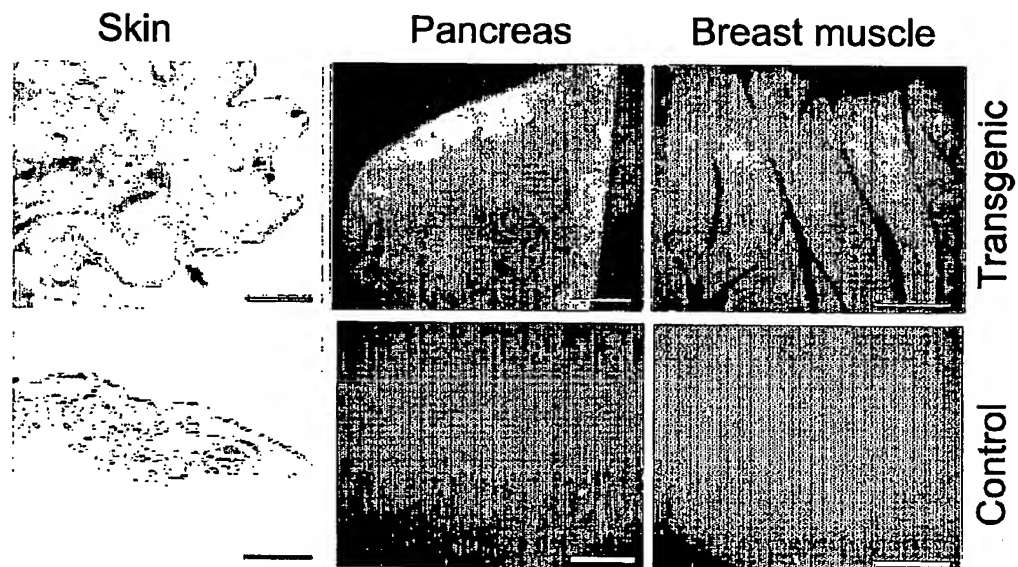


Fig. 3a

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*Fig. 3b**Fig. 3c*

5 / 9

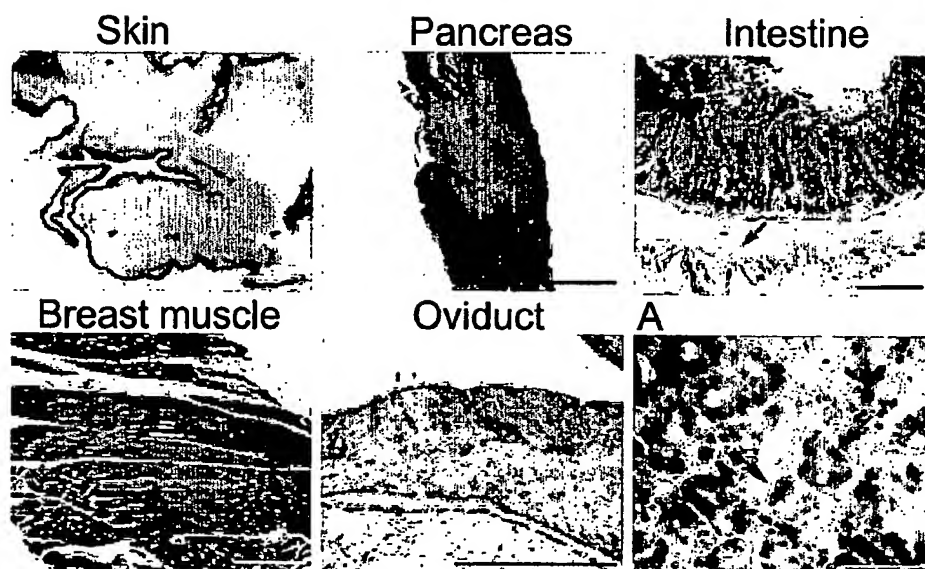


Fig. 4a

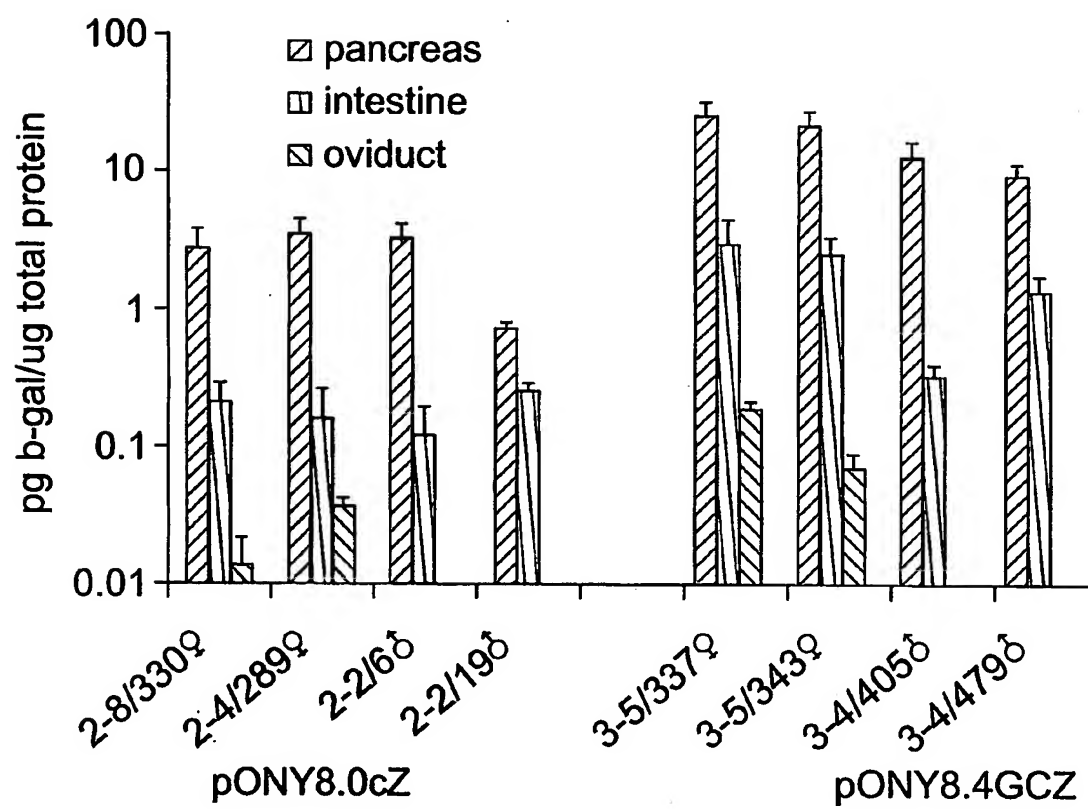


Fig. 4b

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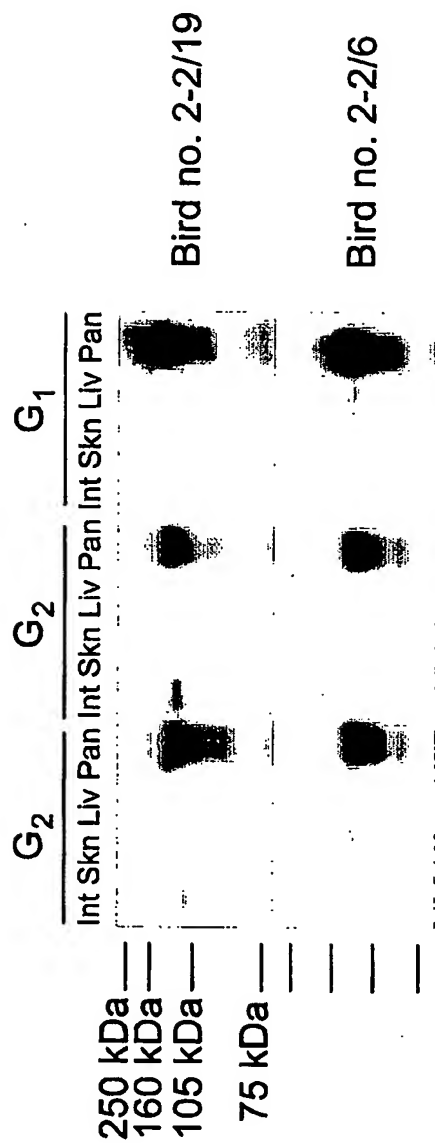


Fig. 5a



Fig. 5b

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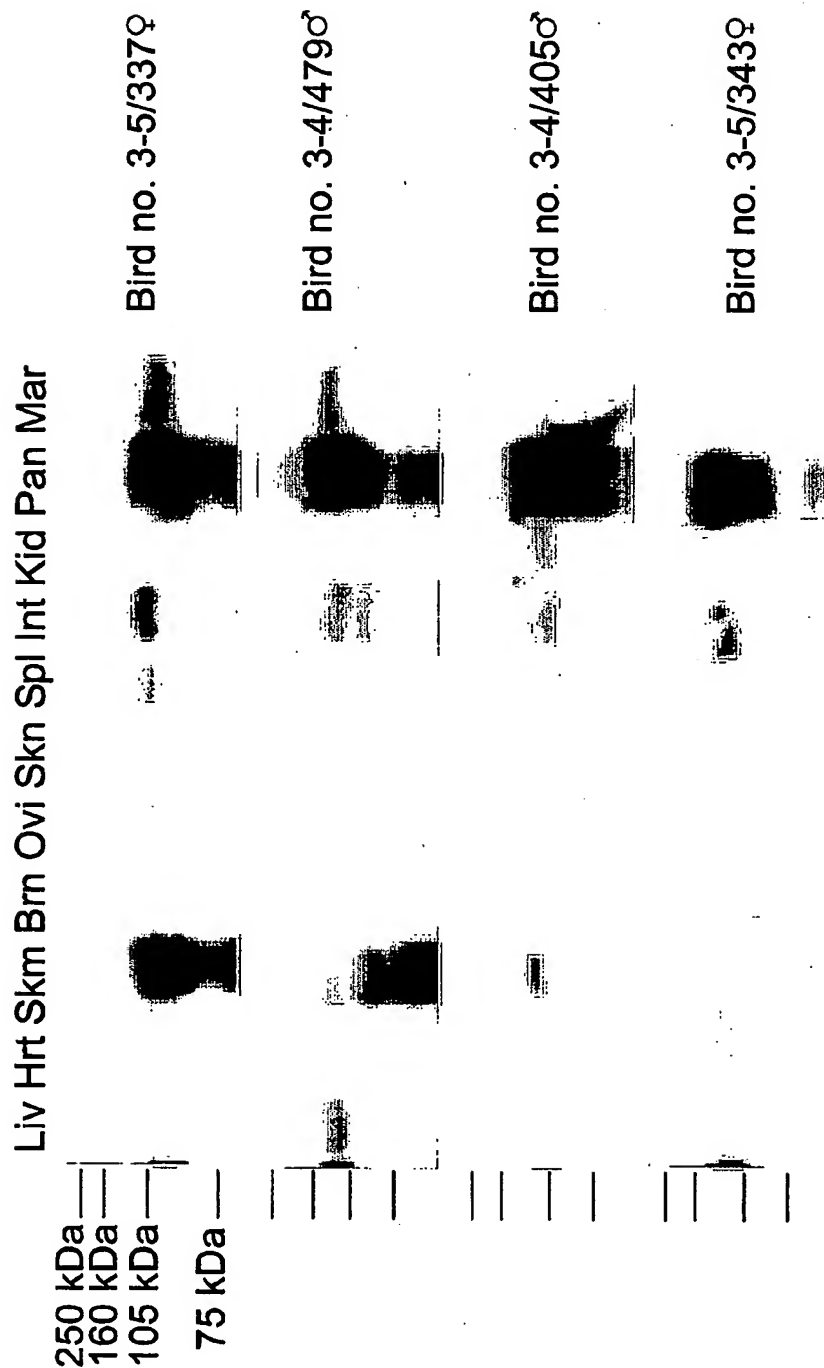


Fig 6

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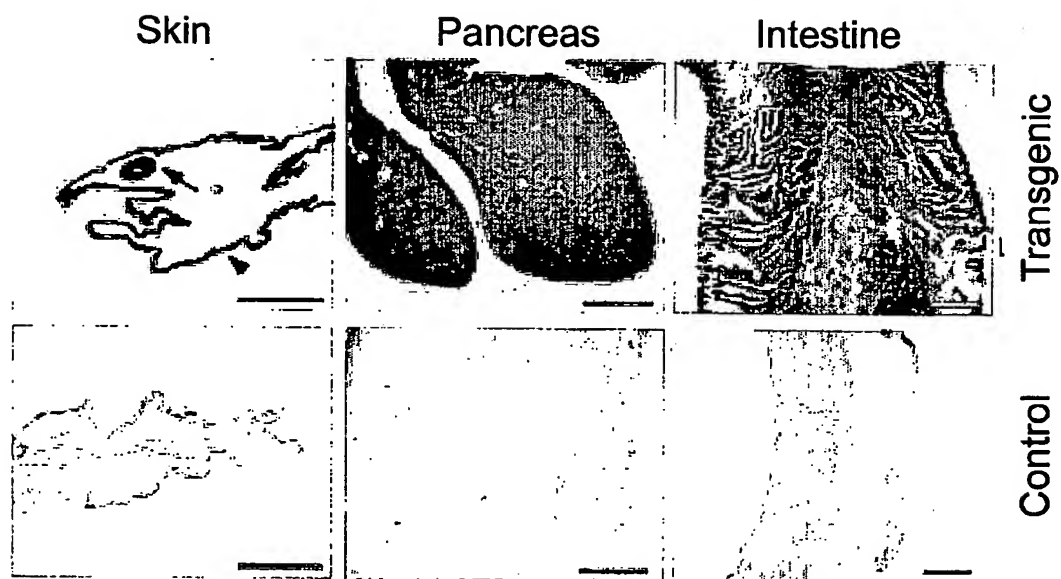


Fig 7

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pONY8.4GCZ
expression in
oviduct

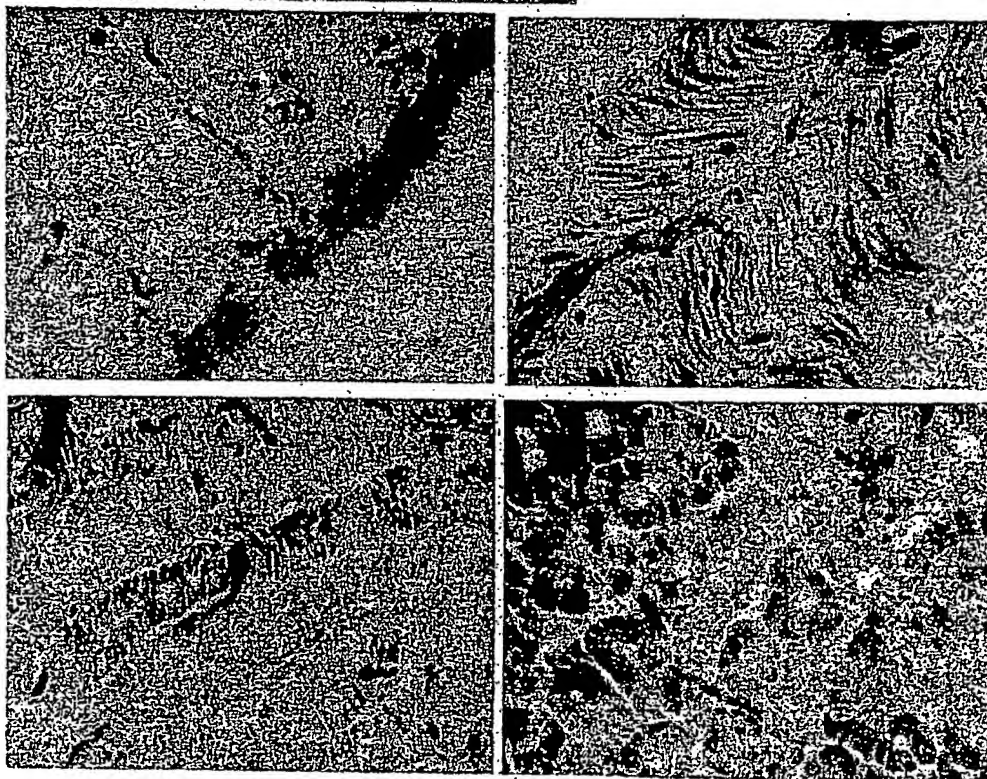


Fig. 8